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Protection against *Vibrio cholerae* El Tor Infection by Specific Antibodies against Mannose-Binding Hemagglutinin Pili

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Both specific polyclonal antiserum and monoclonal antibodies against mannose-binding hemagglutinin fimbriae of *Vibrio cholerae* (mannose-sensitive hemagglutinin [MSHA]) were shown to protect against experimental cholera caused by vibrios of the El Tor biotype in the infant mouse and in the rabbit intestinal loop models. MSHA-specific Fab immunoglobulin fragments were also protective. No protective effect was observed against challenge with *V. cholerae* O1 of the classical biotype. These results suggest that MSHA pili play an important role in the pathogenesis of cholera caused by the El Tor biotype of *V. cholerae* and that induction of intestinal anti-MSHA immunity may be a worthwhile additional objective in the development of oral cholera vaccines.

Vibrio cholerae O1 is the causative agent of epidemic cholera. *V. cholerae* exists as two biotypes, classical and El Tor, and as two main serotypes, Inaba and Ogawa. Cholera disease is caused by the action of cholera toxin, which through its stimulation of intestinal adenylate cyclase activity gives rise to the characteristic excessive electrolyte secretion and fluid loss from the small intestine. However, colonization of the small intestine by *V. cholerae* strains is also an essential step in the pathogenesis. Different putative adhesive factors have been proposed to be involved in colonization (5–7, 14, 15). One of these, a toxin-coregulated pilus has been shown to be important for colonization of *V. cholerae* O1 of the classical biotype (9, 17, 18, 21). The importance of the toxin-coregulated pilus as a colonization factor for vibrios of the El Tor biotype has been questioned (7, 14, 18); it has been suggested that there are colonization factors other than the toxin-coregulated pilus that may be involved in colonization of the intestine by *V. cholerae*, especially of the El Tor biotype (3, 6, 8, 15). One possible candidate may be the cell-associated hemagglutinin which is sensitive to D-mannose (MSHA) (11, 13). This hemagglutinin is associated with the El Tor biotype and is strongly immunogenic (10). Recently, we showed that MSHA is associated with pili that are expressed on the surface of most or all strains of El Tor bacteria grown both in vitro and in vivo during cholera infection (12).

The aim of these studies was to determine whether specific antibodies to MSHA (polyclonal serum as well as monoclonal antibodies [MAb]) can protect against experimental El Tor cholera. This was analyzed in passive protection tests with two different animal models, infant mice and rabbit intestinal loop tests.

Two *V. cholerae* O1 El Tor strains (174 and T19479, both Inaba serotype) and two classical strains (395 Ogawa and X28214 Inaba) were used. Bacteria were cultured in Trypticase soy broth without glucose at 37°C for 4 to 6 h, conditions shown to lead to high levels of expression of MSHA fimbriae (13). The bacteria were then harvested by centrifugation and washed twice with phosphate-buffered saline (PBS).

An antiserum to MSHA was prepared in rabbits by immunization with a crude MSHA preparation as described previously (20). The antiserum was then extensively absorbed with MSHA-negative bacteria (both boiled and live) of the classical biotype but of the homologous serotype. The antibody titer of the absorbed serum against crude MSHA remained high, 110,000 as tested by an enzyme-linked immunosorbent assay (ELISA) (20), while the ELISA antilipopolysaccharide (LPS) titer dropped from 300,000 to 400 and the vibriocidal titer disappeared completely as the result of the absorption. A MAb to MSHA, 17:10 (immunoglobulin G3 [IgG3]), was prepared and characterized as described previously (12, 20); ascites fluid that contained 27 mg of IgG3 per ml was produced by inoculating the hybrid cells into the peritoneal cavities of mice.

The specificity of the polyclonal antiserum and MAb 17:10, respectively, for MSHA has been amply documented by both functional and morphological criteria as described previously (12, 20). The specificity of these immune reagents was further established in immunoblot analyses performed as described previously (12) using a crude MSHA preparation separated in a 17% polyacrylamide gel and subsequently transblotted to nitrocellulose paper essentially as described (22). After being blocked with 1% bovine serum albumin-PBS, the nitrocellulose sheets were incubated for 2 h with antibodies and then incubated with anti-Ig horseradish peroxidase conjugates as secondary antibodies (Jackson Immuno Research Laboratories, West Grove, Pa.) and H_2O_2 with 4-chloro-1-naphthol (Bio-Rad Laboratories, Richmond, Calif.) as the enzyme substrate. As shown in Fig. 1, the 17-kDa MSHA protein band was specifically stained by antibodies of the absorbed and nonabsorbed rabbit anti-MSHA serum as well as by the anti-MSHA MAb. No staining of the MSHA band was obtained either with preimmune serum or with nonspecific MAb.

Fab fragments of rabbit antibodies against MSHA were prepared essentially as described by Porter (16). The antiserum was precipitated with ammonium sulfate added to 50% saturation, and after centrifugation (13,000 × g for 10 min),

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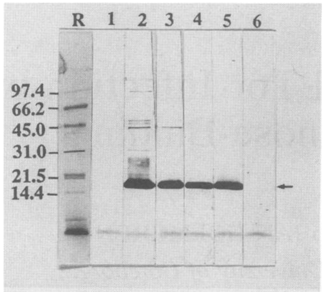

FIG. 1. Immunoblot analysis showing specificity of anti-MSHA antibodies used in the passive protection tests. The transblotted MSHA antigen was developed with different antibody preparations: rabbit preimmune serum (lane 1); rabbit MSHA unabsorbed antiserum (lane 2); MSHA antiserum absorbed with MSHA-negative vibrios (lane 3); Fab fragments of the absorbed MSHA antiserum (lane 4); anti-MSHA MAb 17:10 (lane 5); nonspecific MAb (lane 6). All antibody preparations were tested in comparable Ig concentrations: sera and MAbs, 1:2,000, and Fab fragments, 1:500. Molecular mass markers (in kilodaltons) were amido black stained (lane R). The position of MSHA (17 kDa) is shown by an arrow.

the pellet was extensively dialyzed against 0.1 M sodium phosphate buffer (pH 7.0). A solution containing 5.8 mg of Ig per ml was treated with papain attached to agarose beads (Sigma Chemical Co., St. Louis, Mo.; final concentration, 1.25 mU/ml) suspended in 0.1 M phosphate buffer (pH 7.0) with cysteine-HCl (final concentration, 10 mM) and EDTA (final concentration, 2 mM) at 37°C for 16 h as described previously (1). After centrifugation, the supernatant was filtered through a G25M Sephadex PD10 column (Pharmacia, Uppsala, Sweden).

The capacity of anti-MSHA antibodies to protect against experimental cholera was evaluated in infant mice (2) and in rabbit ileal loop (19) tests. For the infant mouse experiments, 3- to 5-day-old Swiss mice that were separated from their mothers and starved for 4 to 5 h before the challenge were used. Bacteria of the respective strain were adjusted to a concentration of 10^{10} cells per ml, centrifuged ($13,000 \times g$ for 5 min), and resuspended to the same volume with the respective antibody preparation or for control purposes with a nonspecific MAb or preimmune serum. After incubation at room temperature for 1 h, the bacterium-antibody mixtures were administered (in a 50-μl volume, i.e., 5×10^8 vibrios per mouse) into the stomach via the mouth with an eye needle (Unimed, Lausanne, Switzerland) and a syringe. Groups of mice with 6 to 16 animals in each group were given the respective bacterium-antibody mixture or bacteria-preimmune serum (or nonspecific MAb). Thereafter, the mice were inspected several times daily for diarrhea and death. The protective effect of specific antibodies was determined by mouse survival after 48 h in groups receiving specific anti-MSHA antibodies compared with groups receiving the same bacterial challenge with nonspecific antibodies (or preimmune serum). Protective efficacy was calculated as follows: [1 − (rate ratio of dead mice in the presence of MSHA antibodies versus in the presence of unspecific antibodies)] × 100 (4).

The rabbit intestinal loop model was performed essentially as described previously (1, 19). Serial 10-fold dilutions of bacteria (10^6 to 10^8 cells per ml) were mixed with a fixed concentration of the respective antibody preparation or corresponding preimmune serum or non-related MAb; after

TABLE 1. Protective effect of antibodies to MSHA pili against experimental El Tor but not classical *V. cholerae* infection in the infant mouse cholera model

Antibody prepn (dilution)	ELISA titer[a] against:		Survivors/total (% PE)[b]	
	MSHA	LPS	El Tor	Classical
Polyclonal antiserum:				
MSHA (1:50)	2,200	8	10/12* (76)	2/6** (33)
Preimmune (1:50)	3	<1	5/16	0/6
MAb				
MSHA (1:30)	1,400	<1	8/12*** (92)	1/6
Nonspecific (1:30)	<1	<1	1/12	1/6

[a] Titers in antiserum or MAb dilution used in the test.

[b] Survivors were from the total number challenged as determined after 48 h; results are based on three separate experiments. PE, protective efficacy determined as described in text. *, $P = 0.0083$; **, $P = 0.23$; ***, $P = 0.0047$ (versus preimmune serum or nonspecific MAb [Fisher's exact test]).

incubation at room temperature for 1 h, the mixtures were injected into 5-cm small bowel loops in 1-ml volumes. Each preparation was injected into randomly positioned loops in at least three different rabbits. After 18 to 20 h, the animals were sacrificed and the ratio between fluid accumulation and length of the loop (milliliters per centimeter) was calculated. For each animal, the dose of the challenge strain needed to induce half-maximal fluid accumulation (50% effective dose [ED_{50}]) in the loops in the presence of anti-MSHA and nonspecific antibodies, respectively, was determined. The protection factor (PF) was then calculated as the ratio between the geometric mean of individual ED_{50} values in the presence of specific and nonspecific antibodies as follows: PF = mean ED_{50} in anti-MSHA antibodies/mean ED_{50} in nonspecific antibodies (1).

Statistical analyses were done by Fisher's exact test.

The results from the infant mouse studies indicate that the antibodies against MSHA had a protective effect against challenge with *V. cholerae* O1 bacteria of the El Tor but not the classical biotype. Protection against El Tor (strain 174) was observed with either absorbed polyclonal antiserum or MAb (Table 1); the effect was superior to that obtained with preimmune serum or MAb of irrelevant specificity. Although the MSHA preparation used to raise the polyclonal antiserum was not pure, the protection obtained with the absorbed antiserum was most likely due to specific antibodies against MSHA pili. Before use, the rabbit antiserum had been extensively absorbed with MSHA-negative bacteria so that, as specified above, the ELISA anti-LPS titer of the serum had been reduced 750-fold and no detectable vibriocidal activity remained. Furthermore, the absorbed antiserum had no protective effect against challenge with classical *V. cholerae* strains of homologous serotype that do not express MSHA (12, 13) (Table 1). The protective effect of MSHA antibodies was further supported by the finding that MAb 17:10 substantially reduced mortality in the mice infected with El Tor but not in mice infected with classical vibrios. The MAb ascites fluid used also lacked vibriocidal activity and did not react against LPS in ELISA although it reacted strongly with MSHA in ELISA (Table 1) and immunoblot tests (Fig. 1).

To determine whether the protective effect of MSHA antibodies against experimental El Tor cholera was due to specific blocking of fimbrial attachment to the intestinal epithelium preventing colonization or to bacterial agglutina-

TABLE 2. Fab preparation of anti-MSHA Ig protects against experimental El Tor cholera infection in the infant mouse cholera model

Antibody prepn (dilution)	MSHA ELISA titer[a]	Survivors/total[b]	PE (%)[c]
Fab fragments of anti-MSHA (1:12)	1,800	8/11*	64
Total anti-MSHA (1:50)	2,200	4/4**	100
Preimmune serum (1:50)	3	4/15	

[a] Titers in dilution of antiserum used in the test.
[b] Survivors were from the total number challenged as determined after 48 h. *, $P = 0.026$; **, $P = 0.018$ (versus preimmune serum [Fisher's exact test]).
[c] PE, protective efficacy determined as described in text.

tion, we prepared Fab fragments of the polyclonal anti-MSHA serum and tested them for protective effect against El Tor challenge in infant mice. The fragments did not agglutinate MSHA-positive bacteria but were able to inhibit hemagglutination by MSHA-expressing bacteria and also reacted strongly with the MSHA subunit in Western immunoblots (Fig. 1, lane 4). When tested in an Ig concentration corresponding to that of the MSHA antiserum, the Fab fragments were also found to offer significant protection against challenge with the El Tor strain 174 (Table 2). Protective efficacies in the presence of Fab fragments and total anti-MSHA antiserum were 64 and 100%, respectively, compared with that in the presence of the preimmune serum.

The results from the infant mouse cholera model were confirmed when the protective effect of antibodies against MSHA was tested in the rabbit ileal loop model. This was done by evaluating the capacity of the anti-MSHA rabbit serum and MAb 17:10 to inhibit fluid secretion in ileal loops challenged with the different El Tor and classical *V. cholerae* strains (Table 3). It was shown that the polyclonal antiserum had a very strong protective effect against challenge with bacteria of the El Tor biotype, while no such effect was seen when classical strains were tested. Thus, the dose of the different challenge strains (i.e., 174 and T19479) giving half-maximal fluid accumulation (ED_{50}) in the loops increased 14 and 80 times, respectively, in the presence of rabbit antiserum to MSHA fimbriae compared with that in preimmune serum. When the MAb was used, the protective effect was less pronounced, with 3.5- and 2-fold-increased ED_{50} values in the presence of the specific MAb compared with the nonspecific MAb; the serum and MAb were not protective against two classical strains tested (Table 3).

Using two different animal cholera infection models, we found that a MAb and an extensively absorbed antiserum against MSHA pili were able to protect animals against challenge with El Tor but not with classical *V. cholerae* strains. This is analogous to results from a previous study (12) showing that MSHA fimbriae are usually only expressed on the surface of *V. cholerae* of the El Tor biotype. In the present study, we demonstrated that antibodies to MSHA fimbriae may decrease fluid secretion in the gut caused by *V. cholerae* El Tor strains as tested in the rabbit intestinal loop model. However, in these intestinal loops in which bacteria are not mechanically removed by peristalsis, adhesion might not be necessary for the virulence of *V. cholerae*. Therefore, we also used the infant mouse cholera model, which allows study of the protective effect of antibodies against infection and death in a nonligated intestine. A strong protective effect against *V. cholerae* El Tor challenge was observed in this model when using the anti-MSHA serum diluted as much as 1:50. The observed strong protective effect of the Fab fragments supports our notion that anti-MSHA antibodies may mediate protection in experimental cholera by blocking bacterial adherence to the intestinal epithelial cells. Furthermore, the finding that MAb 17:10 was protective supports the idea that antibodies to MSHA pili indeed may mediate protection against experimental cholera caused by El Tor vibrios.

Chaicumpa and Atthasishtha (3) showed that rabbit antiserum to *V. cholerae* El Tor hemagglutinins was able to protect infant mice against experimental cholera. However, neither the anti-LPS titer nor the vibriocidal activity of this serum was tested. Therefore, it is not clear to what extent the protective effect of the antiserum used by Chaicumpa and Atthasishtha (3) was due to antibodies to LPS rather than against El Tor hemagglutinins. To avoid any risk of contaminating anti-LPS antibodies in our system, we tested MAbs to MSHA in addition to extensively LPS-absorbed polyclonal antiserum.

The results of our study support the idea that MSHA pili may play an important role in the pathogenesis of experimental cholera caused by *V. cholerae* El Tor since specific antibodies directed to MSHA antigen were able to protect against challenge with El Tor but not with classical strains. This suggests that the protective efficacy of a recently developed oral cholera vaccine, the so-called B subunit-whole cell vaccine (4), against El Tor cholera may be enhanced by including MSHA pili in future generations of this vaccine.

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TABLE 3. Protective effect of antibodies to MSHA pili against experimental El Tor *V. cholerae* infection in the rabbit ileal loop test

Antibody prepn (dilution)	Challenge with strain:							
	El Tor				Classical			
	174		T19479		395		X28214	
	ED_{50} (10^6)	PF[a]	ED_{50} (10^6)	PF	ED_{50} (10^6)	PF	ED_{50} (10^6)	PF
MSHA antiserum (1:50)	22	14	80	80	0.5	1	1	1.2
MSHA MAb (1:30)	5.6	3.5	2	2	0.6	1.2	0.9	1.1
Control[b]	1.6		1		0.5		0.8	

[a] A PF value of >1.5 is regarded as significant.
[b] Rabbit preimmune serum (1:50) or nonspecific MAb (1:30).

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REFERENCES

1. **Åhren, C. M., and A.-M. Svennerholm.** 1982. Synergistic protective effect of antibodies against *Escherichia coli* enterotoxin and colonization factor antigens. Infect. Immun. **38:**74–79.
2. **Baselski, V., R. Briggs, and C. Parker.** 1977. Intestinal fluid accumulation induced by oral challenge with *Vibrio cholerae* or cholera toxin in infant mice. Infect. Immun. **15:**704–712.
3. **Chaicumpa, W., and N. Atthasishtha.** 1979. The study of intestinal immunity against *V. cholerae*: purification of *V. cholerae El Tor* haemagglutinin and the protective role of its antibody in experimental cholera. Southeast Asian J. Trop. Med. Public Health **10:**73–80.
4. **Clemens, J. D., D. A. Sack, J. R. Harris, F. van Loon, J. Chakraborty, F. Ahmed, M. R. Rao, M. R. Khan, M. D. Yunus, N. Huda, B. N. Stanton, B. A. Kay, S. Walter, R. Eeckels, A.-M. Svennerholm, and J. Holmgren.** 1990. Field trial of oral cholera vaccines in Bangladesh: results from three-year follow-up. Lancet **335:**270–273.
5. **Ehara, M., M. Ishibashi, Y. Ichinose, M. Iwanaga, S. Shimotori, and T. Naito.** 1987. Purification and partial characterization of fimbriae of *Vibrio cholerae* O1. Vaccine **5:**283–288.
6. **Finn, T. M., J. Reiser, R. Germanier, and S. J. Cryz, Jr.** 1987. Cell-associated hemagglutinin-deficient mutant of *Vibrio cholerae*. Infect. Immun. **55:**942–946.
7. **Hall, R. H., P. A. Vial, J. B. Kaper, J. J. Mekalanos, and M. M. Levine.** 1988. Morphological studies on fimbriae expressed by *Vibrio cholerae* O1. Microb. Pathog. **4:**257–265.
8. **Hanne, F., and R. A. Finkelstein.** 1982. Characterization and distribution of the hemagglutinins produced by *Vibrio cholerae*. Infect. Immun. **36:**209–214.
9. **Herrington, D. A., R. H. Hall, G. Losonsky, J. J. Mekalanos, R. K. Taylor, and M. M. Levine.** 1988. Toxin, toxin-coregulated pili, and the *tox*R regulon are essential for *Vibrio cholerae* pathogenesis in humans. J. Exp. Med. **168:**1487–1492.
10. **Holmgren, J., and A.-M. Svennerholm.** 1983. Cholera and the immune responses. Prog. Allergy **33:**106–119.
11. **Holmgren, J., A.-M. Svennerholm, and M. Lindblad.** 1983. Receptor-like glycocompounds in human milk that inhibit classical and El Tor *Vibrio cholerae* adherence (hemagglutination). Infect. Immun. **39:**147–154.
12. **Jonson, G., J. Holmgren, and A.-M. Svennerholm.** 1991. Identification of a mannose-binding pilus on *Vibrio cholerae* El Tor. Microb. Pathog. **11:**433–441.
13. **Jonson, G., J. Sanchez, and A.-M. Svennerholm.** 1989. Expression and detection of different biotype-associated cell-bound haemagglutinins of *Vibrio cholerae* O1. J. Gen. Microbiol. **135:**111–120.
14. **Jonson, G., A.-M. Svennerholm, and J. Holmgren.** 1990. Expression of virulence factors by classical and El Tor *Vibrio cholerae in vivo* and *in vitro*. FEMS Microbiol. Ecol. **74:**221–228.
15. **Manning, P. A.** 1991. Molecular analysis of potential adhesions of *Vibrio cholerae* O1, p. 139–145. *In* T. Wadström, P. H. Mäkelä, A.-M. Svennerholm, and H. Wolf-Watz (ed.), Molecular pathogenesis of gastrointestinal infections. Plenum Press, New York.
16. **Porter, R. R.** 1959. The hydrolysis of rabbit immunoglobulin and antibodies with crystalline papain. Biochem. J. **73:**119–126.
17. **Sharma, D. P., C. Thomas, R. H. Hall, M. M. Levine, and S. R. Attridge.** 1989. Significance of toxin-coregulated pili as protective antigens of *Vibrio cholerae* in the infant mouse model. Vaccine **7:**451–456.
18. **Sun, D., D. M. Tillman, T. N. Marion, and R. K. Taylor.** 1990. Production and characterization of monoclonal antibodies to the toxin coregulated pilus (TCP) of *Vibrio cholerae* that protect against experimental cholera in infant mice. Serodiagn. Immunother. Infect. Dis. **4:**73–81.
19. **Svennerholm, A.-M.** 1975. Experimental studies on cholera immunization. IV. The antibody response to formalinized *V. cholerae* and purified endotoxin with special reference to protective capacity. Int. Arch. Allergy **49:**434–452.
20. **Svennerholm, A.-M., G. Jonson, and Y. Chang.** 1991. A method for studies of an El Tor-associated antigen of *Vibrio cholerae* O1. FEMS Microbiol. Lett. **79:**179–186.
21. **Taylor, R. K., V. L. Miller, D. B. Furlong, and J. J. Mekalanos.** 1987. Use of *pho*A gene fusion to identify a pilus colonization factor coordinately regulated with cholera toxin. Proc. Natl. Acad. Sci. USA **84:**2833–2837.
22. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.